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Pro-apoptotic signaling in neuronal cells following iron and amyloid beta peptide neurotoxicity

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Abstract

In a previous report, we characterized several oxidative stress parameters during the course of amyloid beta (Aβ) peptide/Fe^{2+}-induced apoptotic death in neuronal cells. In extending these findings, we now report a marked decrease in protein kinase C (PKC) isoforms, reduced Akt serine/threonine kinase activity, Bcl 2-associated death promoter (BAD) phosphorylation and enhanced p38 mitogen-activated protein kinase (MAPK) and caspase-9 and -3 activation, 12 h after addition of both 5 μM Aβ and 5 μM Fe^{2+}. These activities reminiscent for a pro-apoptotic cellular course were blocked in the presence of the iron chelator deferroxamine. Aβ alone, increased PKC isoform levels between three- and four-fold after 12 h, enhanced Akt activity approximately eight-fold and Ser136 BAD phosphorylation two-fold, suggesting that by itself is not toxic. Fe^{2+} alone transiently enhanced p38 MAPK and caspase-9 and -3 enzymes indicative for cell damage, but was not sufficient to cause cell death as previously indicated. GF, a PKC inhibitor or wortmannin, a blocker of the Akt pathway enhanced Aβ/Fe^{2+}-induced toxicity, while SB, a p38 MAPK inhibitor, prevented cell damage and apoptosis. These findings further support the hypothesis that metal ion chelation and inhibitors of pro-apoptotic kinase cascades may be beneficial for Alzheimer's disease therapy.

Keywords: Alzheimer's disease, iron, neuronal cell death, oxidative stress, PKC, signal transduction.

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The amyloid β (Aβ) peptide, a principal component of the amyloid plaque, has been proposed central to the pathogenesis of Alzheimer's disease (AD; Katzman and Saitoh 1991; Selkoe 1994; Braak and Braak 1997). While Aβ deposits were found significantly within neuritic plaques (Glenner and Wong 1984; Selkoe 1996), some studies point out that physiological levels of Aβ or related derivatives may play important roles as normal cellular modulators (Whitson *et al*. 1990; Seubert *et al*. 1992; Koo *et al*. 1993) and in rescuing neurons from excitotoxic injuries (Mattson *et al*. 1993; Mattson 1996, 1997; Harkany *et al*. 2000).

Recent emerging evidence suggests that the choice between either a physiological or a pathological effect of the peptide is determined by the excess presence of transition metal ions (Lynch *et al*. 2000; Gnjec *et al*. 2002). Interaction of the latter with Aβ was proposed to accelerate peptide aggregation and initiate an action as a hydrogen peroxide generator (Cotman 1997; Atwood *et al*. 1999; Smith *et al*. 2000). Transition metal ions such as copper, iron and zinc are the potential candidates to induce the neurotoxic action of the peptide (Hensley *et al*. 1994; Huang *et al*. 1999; Cuajungco *et al*. 2000; Cherny *et al*. 2001) and are presumably released from metalloproteins under stressful conditions. Thus, while the exact molecular basis of the Aβ peptide functions remains ambiguous, a growing body of evidence indicates that environmental stress such as that caused by oxidative stress may be responsible for Aβ-enhanced toxicity and age-related disorders including AD (Butterfield *et al*. 2001; Rottkamp *et al*. 2001).

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Abbreviations used: AD, Alzheimer's disease; Aβ, amyloid-β peptide; Akt, viral oncogene (*v-act*) serine/threonine kinase; BAD, Bcl 2-associated death promoter; DFe, deferroxamine; DTT, dithiothreitol; DMEM/H, Dulbecco's modified Eagle's medium in Hepes buffer; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; GF, GF109203X; MAPK, mitogen activated protein kinase; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PI3K, phosphatidylinositol-3-OH kinase; p38 MAPK, p38 mitogen-activated protein kinase; PKC, protein kinase C; SB, SB203580; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling; PAGE, polyacylamide gel electrophoresis; PBS, phosphate-buffered saline, SDS, sodium dodecyl sulfate.

There is little information about the nature of signaling cascades activated by the interaction of the Aβ peptide or its putatively associated metal ions with the cell membrane. Both indirect and direct evidence derived from experimental animal models, cell cultures or AD tissue specimens suggest that multiple cascades may be involved (Wyss-Coray *et al*. 2001). For example, of the various signaling cascades believed to participate in AD pathophysiology, the Ca^{2+}-dependent and -independent serine/threonine protein kinase C (PKC) has received much attention. This is largely because PKC participates in the α-secretase-mediated cleavage of the amyloid precursor protein (APP) to produce the nonamyloidogenic peptide that is essential for normal Aβ processing regulation (Mills and Reiner 1999; Zhu *et al*. 2001; Mudher *et al*. 2001; Rossner *et al*. 2001). Soluble Aβ has been shown to activate several PKC isoforms (Chauhan *et al*. 1991; Luo *et al*. 1997) and induce PKC-dependent phosphorylation and membrane translocation of the mirystoyl-alanine-rich C-kinase substrate (MARCKS). The latter is one of the major downstream substrates of PKC (Nakai *et al*. 2001; Arbuzova *et al*. 2002). Soluble Aβ present during anoxic/reperfusion episodes caused a biphasic modulation of PKC and enhanced MARCKS phosphorylation in cultured neuronal cells (Kuperstein *et al*. 2001). Reported losses of PKC in discrete areas of the AD brain have been associated with oxidative stress markers and Aβ peptide deposits as well as with memory deficits and dementia (Cole *et al*. 1988; Masliah *et al*. 1990; Olariu *et al*. 2001; Clark *et al*. 1991 Matsushima *et al*. 1996).

The serine/threonine kinase Akt is a key enzyme in the phosphatidylinositol-3-OH kinase (PI3K) cascade that regulates neuronal cell survival and activates several anti-apoptotic proteins in short known as CREB, IKK, Bcl2, while inhibiting pro-apoptotic factors such as BAD, caspase-9 and forkhead (Dudek *et al*. 1997; Yuan and Yankner 2000; Zhou *et al*. 2000; Brunet *et al*. 2001). Activation of the Akt signaling pathway was found elevated in a variety of neural cells after being rescued from beta-amyloid-induced neurotoxicity (Zhang *et al*. 2001a; Kihara *et al*. 2001; Martin *et al*. 2001). In that context, it is notable that BAD, one of the downstream substrates of Akt phosphorylation, has been shown to be up-regulated in mutant mice overexpressing human APP (Stein and Johnson 2002). To date the outcome of redox metal ions' presence on Akt regulation and BAD phosphorylation in cells has not been clarified.

Unlike Akt, the role of the mitogen-activated protein (MAP) kinase in Aβ peptide signaling has been studied in greater detail (Daniels *et al*. 2001). Work using cell culture models point to a possible linkage between Aβ extracellular signal-regulated kinase (ERK) activation (McDonald *et al*. 1998; Combs *et al*. 1999; Grant *et al*. 1999; Pyo *et al*. 1999; Santiard-Baron *et al*. 1999; Rapoport and Ferreira 2000; Chong *et al*. 2001; Smits *et al*. 2001). Nevertheless there have been very few studies examining the outcome of a combined action of transition metal ions and soluble Aβ on signaling cascades involving members of this superfamily. We recently demonstrated that a combination of Aβ and Fe^{2+} caused a delay in the activation of ERKs (Kuperstein and Yavin 2000). When ERK activation was also accompanied by nuclear translocation, neuronal cell cultures turned susceptible to apoptotic death (Kuperstein and Yavin 2002). Another distinct member of the MAP kinase family, p38 MAP kinase, was shown to be active and present in Aβ deposits of a transgenic mouse model of AD (Savage *et al*. 2002). Its levels increased significantly after ischemic injury in APP over-expressing mice (Koistinaho *et al*. 2002) indicating a possible role in oxidative stress-induced cell damage (Hensley *et al*. 1999).

The limited information concerning the triggering of cellular kinases following the concomitant addition of Aβ peptide and divalent iron, prompted us to examine some of the molecular details of this interaction with particular emphasis on neuronal cell death or cell rescue. Using a number of kinase inhibitors, we now demonstrate that while Aβ peptide alone does not enhance cell toxicity, a combination of Aβ and iron enhances cell death via apoptotic pathways. Using the iron chelator deferroxamine, we provide further evidence for the dependency on iron and suggest an oxidative stress component in stimulating an Aβ-dependent death.

Materials and methods

Primary cultures of rat cerebral cortex neurons

Cerebral hemispheres from 16-day-old rat embryos were dissociated as previously described (Kuperstein *et al*. 2001). Immediately following surgery, brains were rinsed in fresh Dulbecco's modified Eagle's medium containing 10 mM Hepes buffer (DMEM/H) and supplemented with 20% fetal calf serum and 2 mM glutamine. After rinsing, the tissue was gently dissociated in approximately 4 mL of the above medium and sieved through a 50-μm nylon mesh (Sefar, Zurich, Switzerland) stretched over a 60-mm sterile culture dish using a cell scraper. The collected cell suspension was centrifuged at low speed (100 *g*) for 1 min at room temperature. The supernatant was discarded and the cell pellet suspended in 1 mL of DMEM/H per brain. Aliquots of the cell suspension were seeded on poly-L-lysine (MW 78 000, Sigma, St Louis, MO, USA)-precoated culture plates. After cell attachment, medium was changed to DMEM/H supplemented with 0.1% fetal calf serum and a mixture of hormones (10 μg/mL insulin, 4 μg/mL hydrocortisone, 5 μg/mL transferrin, 10 ng/mL glycyl-L-hystidyl-L-lysine, 10 ng/mL somatostatin) and the cells incubated at 37°C in the presence of 5% CO_2 in air and 100% humidity. At designated times 5 μM Aβ peptide (Bachem, Babendorf, Switzerland) in the absence or presence of 5 μM $FeSO_4$ (Sigma) were added to cells. For indicated experiments, cells were supplemented with either 10 μM GF {GF 109203X, 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3yl)-maleimide, Calbiochem, Schwalbach, Germany} a PKC inhibitor, 20 μM wortmannin (Sigma), a PI3K/Akt inhibitors or 20 μM SB

{SB203580, [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole] Calbiochem}, a p38 MAPK inhibitor. Cultures were also subjected to 25 μM deferroxamine (DFe, Sigma). For routine supplement, the Aβ peptide was dissolved in double distilled water immediately prior to addition to cells.

Cell viability by MTT

Cells grown in 24-well poly-L-lysine precoated plates were incubated at 37°C for 2 h with 10 μL of 3-[4,5-dimethyl-thiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT, 5 mg/mL PBS from Sigma) in 0.1 mL medium. Following incubation, medium was discarded and 0.2 mL of 0.04 M HCl in isopropanol was added to each well. After shaking, absorbance was measured in the ELISA reader at 540 nm and 630 nm. MTT values normalized to DNA cellular content were based on a procedure detailed elsewhere (Brand *et al*. 2000) and are means of three cultures from three to four independent experiments.

TUNEL assay and α-tubulin immunostaining

Cells grown on poly-L-lysine-coated glass cover slips were subjected to various treatments and subsequently washed with phosphate buffer saline (PBS) and exposed to 3% formaldehyde in PBS solution for 20 min. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) analysis was done according to the manufacturer protocol using the Apoptosis Detection System, Fluorescein-Kit (Promega). Cells were then immunostained with a monoclonal antibody to α-tubulin (Sigma) and the complex revealed by incubation with CY3-conjugated goat anti-mouse antibody (Jackson Immunoresearch, West Grove, PA, USA). Cells were then washed three times with PBS and mounted on a glass slide using a Moviol 4–88 solution. Preparations were imaged with a LSM 510 microscope using a multi-track configuration with two different excitation wavelengths (excitation 543 nm, filter 560 nm) for CY-3 conjugated antibody and TUNEL staining (excitation 488 nm, filter 505–530 nm). Images of different preparations were acquired using the same laser beam intensity and detector sensitivities. The experiments were repeated on several occasions.

Caspase-9 and -3 enzymatic activities

After various treatments, cells grown on 35 mm poly-L-lysine precoated plates were rinsed with PBS, then collected, centrifuged and the pellet permeabilized with 0.1 mL homogenization buffer consisting of 20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin, 10 μg/mL pepstatin and 25 μg/mL aprotinin and 250 mM sucrose. The lysed cell pellet was incubated at 4°C for 10 min and subsequently centrifuged for 5 min at 15 000 *g*. Caspase-9 enzymatic activity was measured by colorimetry following cleavage of *p*-nitroaniline conjugated LEHD (LEHD-pNA) (Calbiochem). Caspase-3 enzymatic activity was measured by colorimetry following cleavage of *p*-nitroaniline conjugated DEVD (DEVD-pNA) substrate (Bachem). To aliquots of the supernatant, 40 μM LEHD-pNA or DEVD-pNA in caspase assay buffer consisting of 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS), 10 mM DTT, 1 mM EDTA and 10% glycerol was added and final solution incubated for 3 h at 37°C. The released pNA chromophore was quantified by spectrophotometry at 405 nm and caspases activity normalized to mg protein according to Bradford (1986). In some experiments cells were supplemented with caspase-3 inhibitor Z-DEVD-FMK (Calbiochem) or caspase-9 inhibitor Z-LEHD-FMK (Calbiochem) 30 min prior to treatment.

PKC identification

Aliquots of the crude extract of cytosolic and membrane fractions (30 μg protein) were separated by sodium dodecyl sulfate (SDS)−8% polyacrylamide gel electrophoresis (PAGE). After separation the protein bands were transblotted from the gel onto nitrocellulose paper (BA 83, Schleicher and Schuell, Germany). Membranes were subjected to blocking buffer consisting of PBS containing 0.05% Tween 20 (Tween/PBS) and 10% skimmed milk and shaken at room temperature for 1 h. After rinsing, the paper was subjected to polyclonal antibodies for various PKC isoforms (1 : 750 dilution in Tween/PBS) for 1 h at room temperature. Following incubation, the first antibody was removed and the paper blot was rinsed three times for 10 min with a solution consisting of Tween/PBS. A second peroxidase-linked goat anti-rabbit antibody (Sigma) diluted 1 : 16 000 in Tween/PBS buffer was added for 50 min at room temperature. Then the second antibody was removed and the paper blot rinsed three times for 10 min with Tween/PBS. Immunolabeled proteins were visualized with a chemiluminescence (ECL) kit from Amersham according to the manufacturer's specifications.

Akt, BAD and p38 MAPK identification

Cells were grown on 35 mm poly-L-lysine-precoated plates. After treatment, cells were washed three times with cold PBS and once with a buffer composed of 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, and 0.1 mM Na_3VO_4. Cells were permeabilized with the above buffer supplemented with 1% Triton-X 100, 1 mM benzamidine, 2 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL pepstatin and 25 μg/mL aprotinin. The lysate was incubated for 10 min at 4°C and centrifuged at 15 000 *g* for 5 min. Aliquots of the supernatant were applied for chromatography on 10% SDS-PAGE and after separation blotted onto nitrocellulose paper (BA 83, Schleicher and Schuell). The resulting blotted membranes were subjected to blocking buffer consisting of 0.05% Tween 20/PBS and 2% albumin bovine (Sigma). Activated Akt was detected by probing blots with an anti-active Akt antibody (Promega, diluted 1: 2000 in Tween/PBS), activated p38 MAPK was detected by probing blots with an anti-active p38 MAPK antibody (Promega, diluted 1 : 5000 in Tween/PBS) for 1 h. Phosphorylation of BAD at Ser136 was detected by probing blots with an antiphospho-BAD antibody (Oncogene, Boston, MA, USA; diluted 1 : 1000 in Tween/PBS) for 3 h. After washing and staining with a second peroxidase-linked goat anti-rabbit antibody, the blots were developed using the ECL kit. After additional blocking, blots were restained for detection of the total (active and nonactive) Akt, p38 MAPK (Promega diluted 1 : 10 000) and BAD (Oncogene diluted 1: 2000). Blots were then incubated with a polyclonal AP-conjugated goat-antirabbit antibody (Jackson, diluted 1 : 7500) and after washing developed by BCIP/NBT AP substrate.

Scanning

Computer-assisted image analysis was performed on a Silver scanner II (LaCie, USA) and values processed with NIH IMAGE 1.63 software program (National Institutes of Health, Bethesda, MD, USA).

Statistics

Statistical analysis among groups was performed by ANOVA with application of Bonferroni multiple comparison tests using the IN-stats view program. Levels of difference were considered significant at $p < 0.05$.

Results

Effects of Aβ and Aβ/Fe^{2+} addition on cellular signaling

PKC signaling

In a previous report we have documented biphasic kinetics of PKC activation after chronic exposure of brain cell cultures to near physiological concentrations of soluble Aβ peptide (Kuperstein *et al*. 2001). In addition, subjecting these cells to anoxic/reperfusion episodes caused a down-regulation of all PKC isoforms a phenomenon that was also accompanied by cell death. The likelihood that divalent metal ions may exacerbate the stress (Gnjec *et al*. 2002) prompted us to examine in some detail the consequences of exogenous metal ions' addition on PKC levels. Figure 1 depicts the changes in several PKC isoforms of neuronal cells in the presence or absence of micromolar quantities of divalent iron after exposure to Aβ. After 4 h incubation with either Aβ or Fe^{2+} or a mixture of both, a marked elevation of the Ca^{2+}-dependent (α-PKC and β1-PKC) as well as Ca^{2+}-independent (δ-PKC and ϵ-PKC) isoforms in comparison to control levels was noticed (left panels). By 12 h however, a further increase in all PKC isoforms was noted in the presence of Aβ peptide alone (right panels). At this time, the presence of iron either alone or in combination with the peptide caused a drastic reduction in PKC levels. The decrease could be prevented by the addition of 25 μM DFe indicating a crucial role for the iron in this down-regulation. The data indicate a synergistic yet delayed effect of Aβ and Fe^{2+} to down-regulate PKC. It also suggests that the soluble peptide acted very distinctly, unlike the one added in combination with iron, and did not result in cell damage.

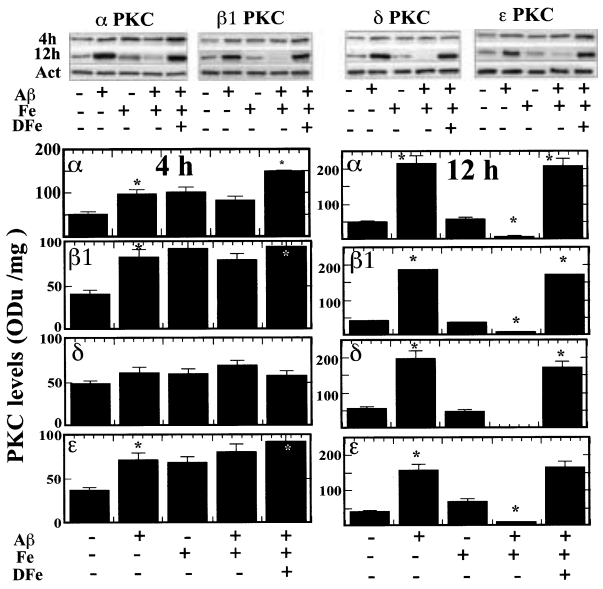

Fig. 1 PKC isoform levels in cerebral cultures after addition of $Aβ_{1–40}$ with or without Fe^{2+}. Dissociated cerebral cortex cells from 16-day-old rat embryos cultured for 1 week under serum-free conditions, were subjected to $Aβ_{1–40}$ (5 μM) or Fe^{2+} (5 μM) or a combination of both for 4 h or 12 h. Deferroxamine (DFe, 25 μM) was added 10 min before adding other components and by itself showed no effect on PKC isoforms profile. After harvesting, cells were homogenized and extracts subjected to SDS gel electrophoresis. After blotting, proteins were stained with polyclonal antibodies to either one of the PKC isoforms and subsequently restained with antibody recognizing β-actin (Act) for gel loading verification. Values obtained from the X-ray films, expressed as arbitrary OD units normalized per mg protein, were collected from two to three separate experiments ± SEM (*$p < 0.001$).

Akt signaling and Ser136 BAD phosphorylability

The time dependent effect of iron, Aβ peptide or a combination of both on Akt regulation is illustrated in Fig. 2. Notable, addition of Aβ to cells induced a rapid activation of Akt after 0.5 h followed by sustained activation up to 12 h. Fe^{2+} supplements resulted in a short-lived Akt activation for 4 h, which declined by 12 h to basal levels. In contrast, a combination of Aβ and Fe^{2+} reduced Akt phosphorylation (compared with 4 h) after an initial rise at 30 min. Addition of DFe restored Akt phosphorylation to levels similar to those induced by Aβ alone. The time course of Akt regulation by Aβ and Fe^{2+} in the presence of DFe, suggests that Fe^{2+} inhibits Aβ-dependent prolonged Akt activation.

As expected, after 12 h incubation, activation of Akt by Aβ nearly doubled Ser136 BAD phosphorylation (Fig. 3). Iron addition slightly reduced (34%, $p < 0.01$) BAD phosphorylation. In contrast a combination of Aβ/Fe^{2+} showed a 71.3% reduction in BAD phosphorylation compared with controls and more than eight-fold compared with Aβ-enhanced levels. This marked inhibition necessitated iron as indicated by the addition of the iron chelator deferroxamine. These results may suggest that amyloid beta peptide-induced activation of the Akt pathway may be part of a physiological signaling of the peptide.

The p38 MAP kinase signaling

Unlike Akt, p38 MAP kinase phosphorylation was practically unchanged after treatment with Aβ compared with controls (Fig. 4). In contrast, a combination of Aβ/Fe^{2+} showed a striking elevation in p38 MAP kinase phosphorylation, reaching a nearly nine-fold higher level compared with basal values by 12 h. This effect was markedly reduced by the presence of deferroxamine. Following Fe^{2+} addition alone, a rapid increase was noticed already at 30 min and

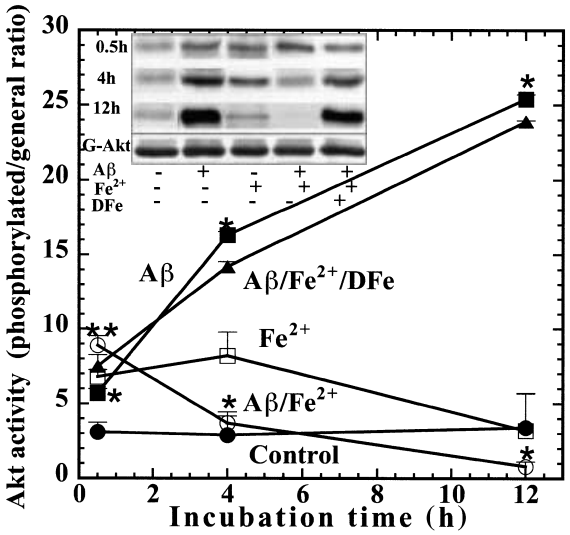

Fig. 2 Effect of Fe^{2+} on $A\beta_{1-40}$-induced Akt phosphorylation in cerebral cultures. Cells were incubated for various time periods with Aβ or Fe^{2+} or a combination of both as detailed in Fig. 1. Deferroxamine (DFe, 25 μM) was added 10 min before adding other components. After blotting, proteins were stained with an antibody to phosphorylated (P) Akt and restained with an antibody to general (G) Akt (12 h blot data). Densitometric values expressed in arbitrary units were collected from three separate experiments ± SEM (*$p < 0.001$; **$p < 0.01$). Insert represents a typical western blot staining of one set of experimental data.

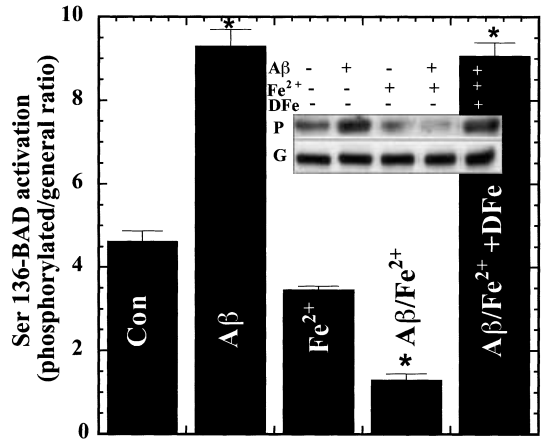

Fig. 3 Effect of Fe^{2+} on $A\beta_{1-40}$-induced Ser136 BAD phosphorylation in cerebral cultures. Cells were incubated for 12 h under similar experimental conditions to those detailed in Fig. 2. Western blots were stained with an antibody specific to phosphorylated (P) BAD and restained with an antibody to general (G) BAD (12 h blot data). Densitometric values expressed in arbitrary units were collected from two to three separate experiments ± SEM (*$p < 0.001$). Insert represents typical western blot staining of one set of experimental data.

remained reasonably high for up to 4 h before decreasing to basal levels. Thus, unlike iron or Aβ, the $A\beta/Fe^{2+}$ combination caused a sustained activation of p38 MAP kinase

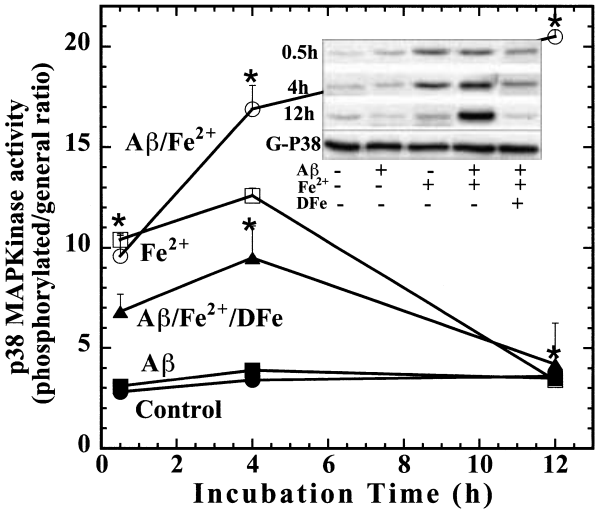

Fig. 4 Effect of Fe^{2+} on $A\beta_{1-40}$-induced p38 MAPK phosphorylation in cerebral neuronal cultures. Experimental conditions were similar to those described in Fig. 2. After blotting, proteins were stained with an antibody specific to the phosphorylated p38 MAPK form and restained with an antibody to general (G) p38 MAPK (12 h blot data). Densitometry values of p38 MAPK expressed in arbitrary units are from three separate experiments ± SEM (*$p < 0.001$). Insert represents a typical western blot staining of p38 MAPK.

notable even after 24 h (data not shown). This indicates that in the absence of Aβ, the effect of iron was transient and unable to unleash a signaling cascade characteristic to initiate cell damage.

Kinase inhibitors and cell toxicity

Modulation of mitochondria activity

Further characterization of the molecular consequences of Aβ and $A\beta/Fe^{2+}$ cotreatments on markers of neuronal cells survival was accomplished by using a number of protein kinase inhibitors. Among these, GF effectively inhibited PKC activity, while wortmannin and SB blocked Akt through PI3 kinase inhibition and p38 MAPK, respectively. As shown in Fig. 5 (left panel), addition of Aβ alone had no effect on the ability of neuronal cells to reduce MTT, an index of general cellular dehydrogenase activity and a marker often used to assess cell viability. A slight reduction (20%) in mitochondrial activity was noticed in the presence of iron alone. A further reduction however (approximately 50%) was observed when the $A\beta/Fe^{2+}$ combination was present in the culture medium. The synergistic effect of the latter was further amplified when either 10 μM GF or 20 μM wortmanin were added to cells (Fig. 5 right panel). Compared with the matched controls, a further reduction of nearly five-fold in MTT reduction assay has been noticed. By 24 h the majority of cells showed morphological signs of toxicity and eventually died (data not shown).

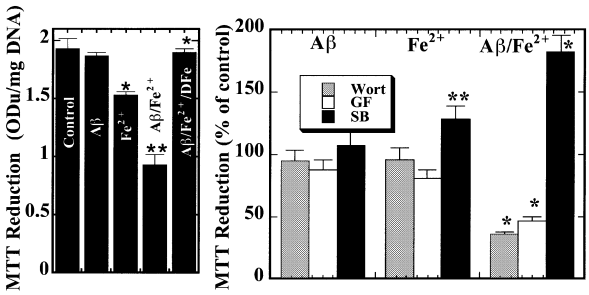

Fig. 5 Effect of various kinase inhibitors on mitochondrial activity following Aβ/Fe^{2+} treatment. MTT reduction assay was performed on the cultured cells treated with either 5 μM Aβ or 5 μM Fe^{2+} or a combination of both for 12 h in the absence (left panel) or presence of the kinase inhibitors wortmannin (Wort), GF and SB each added at 20, 10 and 20 μM, respectively (right panel) and DFe (25 μM). Values expressed as arbitrary OD units normalized per mg cellular DNA in the left panel or as a percentage of the value obtained in the presence of inhibitor but without iron or Aβ (right panel) are average ± SEM of three separate experiments. *$p < 0.001$; **$p < 0.01$, other values were not significant. Cultures containing inhibitors but neither Fe^{2+} nor Aβ were not statistically different from untreated cultures.

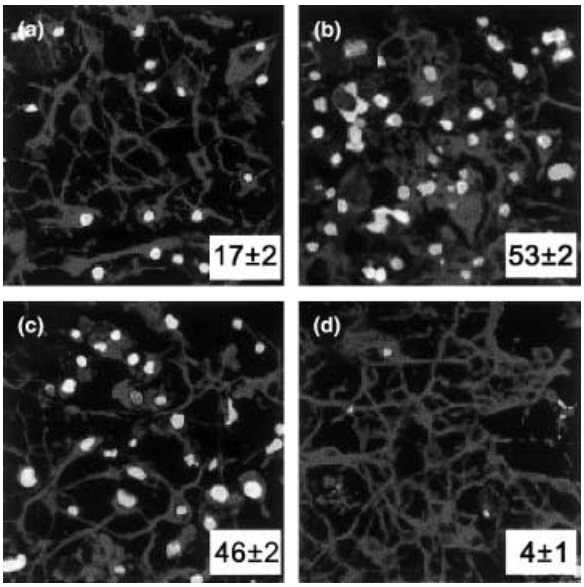

Fig. 6 Effect of kinase inhibitors on Aβ/Fe^{2+}-induced apoptotic cell death. Cerebral cells were grown on glass cover slips and co-stained for DNA fragmentation by TUNEL (green) technique and by an antibody to α-tubulin (red) for cytoskeleton immunovisualization. Observation and photography was done on cells using a confocal laser microscope after 24 h following addition of 5 μM Aβ and 5 μM Fe^{2+} in the absence (a) or presence of either 20 μM wortmannin (b); 10 μM GF (c); or 20 μM SB (d). Numbers in the insert are percent values of TUNEL-positive cells counted from five separate fields. Less than 5% TUNEL-positive cells were detected in cultures treated with either Fe^{2+} or Aβ alone (data not shown).

Addition of GF and Akt inhibitors is in accord with previous studies suggesting a possible protective role of both PKC and Akt in cell survival (Cole *et al*. 1988; Clark *et al*. 1991; Yuan and Yankner 2000; Brunet *et al*. 2001). In contrast, addition of 20 μM SB, a potent p38 MAP kinase inhibitor, rescued cells as also emphasized by the lack of further decrease in MTT reduction activity. Notable, addition of GF and wortmanin to cells incubated in the presence of iron alone did not accelerate, but to a limited extent, the loss of MTT reduction capability of the cultures. In accord with the data shown in Fig. 4 and our previous report (Kuperstein and Yavin 2002), this strongly suggests that Aβ/Fe^{2+}-induced cell damage may act via a p38 MAP kinase signaling cascade.

Modulation of apoptotic cell death

Further evidence that inhibition of cellular rescue pathways involving Akt and PKC on one hand or inhibition of p38 MAP kinase pro-apoptotic signaling on the other hand, prevented cell death, is illustrated by DNA fragmentation as revealed by TUNEL-positive cells (Fig. 6). While the Aβ/Fe^{2+} combination enhanced the number of TUNEL-positive cells (Fig. 6a) both wortmannin (Fig. 6b) or GF (Fig. 6c) caused a dramatic increase in the number of apoptotic cells. In contrast, inhibition of the pro-apoptotic p38 MAPK pathway by SB, protected cells from the combined Aβ/Fe^{2+} toxicity (Fig. 6d). The experiments above are consistent with the working hypothesis that Aβ in combination with Fe^{2+} renders the neuronal cells sensitive to atrophic signaling cascades by interfering with the PKC and Akt activities.

Activation of BAD

A further illustration of the effect of the above inhibitors on the Akt downstream signaling cascades, is notable in the case of Ser136 BAD phosphorylation. As seen in Fig. 7, in the presence of Aβ wortmannin and GF decreased the Aβ-enhanced phosphorylation levels (see Fig. 3) to control levels while inhibition of p38 MAPK by SB had no effect. Iron, which by itself affected by some 30% BAD phosphorylation, enhanced further reduction in the presence of wortmannin and GF. Reduction of BAD phosphorylation attained almost a peak level (80–90%) in all Aβ/Fe^{2+} pretreated cultures supplemented with either GF or wortmannin in accord with the inhibition of the respective PKC and Akt activities. In contrast, inhibition of p38 MAP kinase by SB did not affect the capacity of cells to phosphorylate the Ser136 moiety on the BAD molecule. Furthermore, in the presence of SB an up-regulation in BAD activation notable both in the presence of Aβ/Fe^{2+} or Fe^{2+} alone was evident. For reasons not yet clear, it should be noted that inhibition of PKC and Akt activities by GF and wortmannin, respectively, reduced to normal levels the Aβ-induced BAD activation.

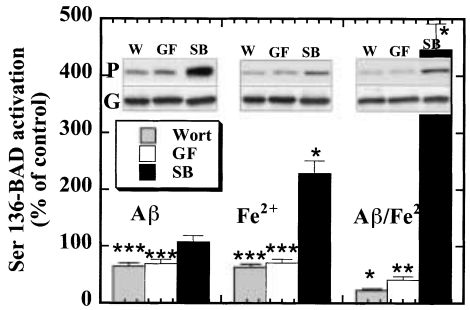

Fig. 7 Effect of kinase inhibitors on Aβ/Fe^{2+}-induced Ser136 BAD phosphorylation. Experimental conditions were identical to those described in Fig. 3. Cultures were also subjected to wortmannin (W) GF, and SB inhibitor for 12 h as detailed in Fig. 5. Cell extracts were subjected to SDS gel electrophoresis and after blotting proteins were stained with an antibody specific to phosphorylated (P) BAD and restained with an antibody to general (G) BAD (12 h blot data). Densitometric values expressed in arbitrary units collected from two to three separate experiments ± SEM (*$p < 0.001$, **$p < 0.01$, ***$p < 0.05$) were normalized to control cells treated with the inhibitor but not with iron or Aβ. Inserts represent typical western blot staining of phosphorylated BAD. Cultures containing inhibitors but neither Fe^{2+} nor Aβ were not statistically different from untreated cultures.

Activation of caspases

In a previous study, we showed that treatment with Aβ/Fe^{2+} enhanced caspase-3 activity, whereas iron removal by chelation with DFe or inhibition of MEK by U0126, reduced it (Kuperstein and Yavin 2002). To examine the possible effects of PKC and Akt inhibitors we have now measured directly caspase-9 and caspase-3 activities under these experimental conditions. As seen in Fig. 8, in the presence of Aβ and Fe^{2+}, both caspase-9 and -3 were activated six- and four-fold, respectively (left panels). A two-fold increase was noticed in caspase activity after Fe^{2+} addition. In contrast, Aβ alone had no effect on either caspase-9 or -3 activities. A partial reduction of caspase activities was found after addition of DFe. Wortmannin and GF enhanced however, caspase-9 and -3 activities in the presence of Aβ most likely because of interference with Akt and PKC signaling cascades, respectively (Fig. 8, righthand panels). As expected, inhibition of the p38 MAPK pathway by SB reduced both caspase-9 and -3 activities. A similar picture was noticed when Fe^{2+} was present alone with either one of the three inhibitors. The elevation of caspase-9 and -3 activities was most pronounced in the presence of the Aβ and Fe^{2+} addition and was further increased following treatment with wortmannin and SB inhibitors.

Discussion

The first aim of this work was to investigate the response and possible interaction between three major cellular phosphokinases, PKC, Akt and p38 MAPK, after exposure of neuronal cells to either soluble Aβ peptide, or to a combination of Aβ and divalent iron. At least the first two of the three kinases noted above are believed to play essential roles in signaling cascades important for cell survival. Taking advantage of inhibitors with a well established specificity to each of these kinases, we have attempted to outline a reciprocal interaction between pro-apoptotic and nonapop-

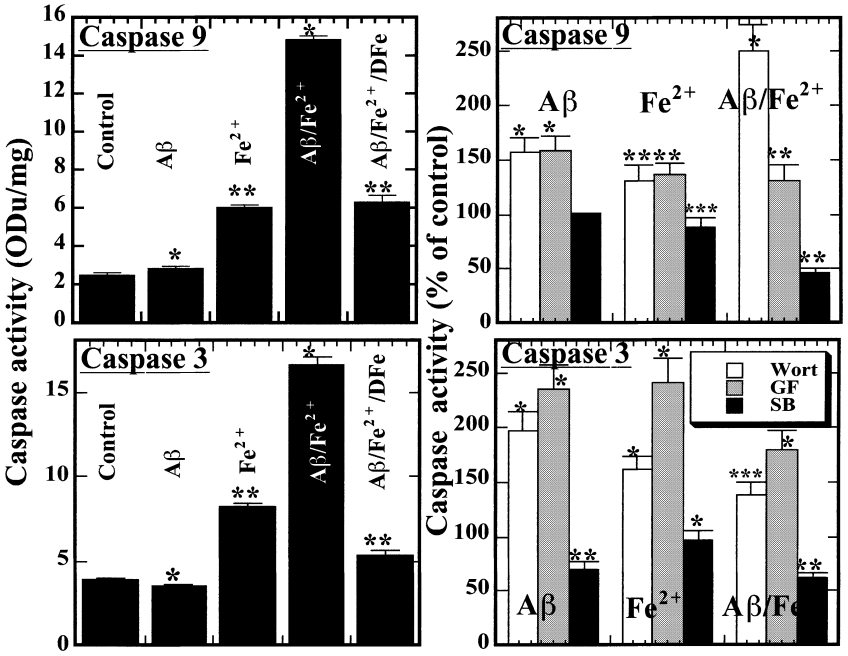

Fig. 8 Effect of various kinase inhibitors on Aβ/Fe^{2+}-induced caspase-9 and -3 activities. Experimental conditions were identical to those detailed in Fig. 5. Cells were treated for 12 h with either 5 μM Aβ or 5 μM Fe^{2+} or a combination of both for 12 h in the absence (left panel) or presence of the kinase inhibitors wortmannin (Wort), GF and SB, each added at 20, 10 and 20 μM, respectively (right panel) and DFe (25 μM). Measurement of the caspase-9 and -3 activity was carried out as detailed under Materials and methods. Values expressed as arbitrary units normalized per mg protein (left panels) or calculated as percent of inhibitor-treated control cultures in the absence of iron and Aβ are average ± SEM (*$p < 0.001$, **$p < 0.01$, ***$p < 0.05$) of triplicate experiments. Cultures containing inhibitors but neither Fe^{2+} nor Aβ were not statistically different from untreated cultures.

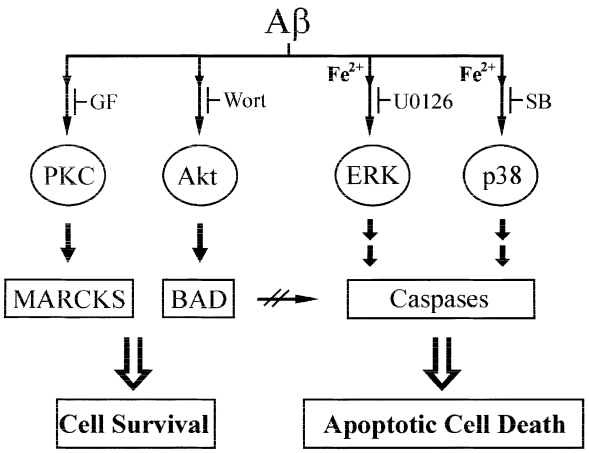

Fig. 9 Activation of apoptotic signaling cascades in cultured cerebral cells by a mixture of Aβ/Fe and lack thereof by Aβ alone. PKC, protein kinase C and its inhibitor GF; Akt, viral oncogene (*v-act*) serine/threonine kinase; and its inhibitor wortmannin; ERK, extracellular regulated kinase and its upstream inhibitor U0126; p38, mitogen activated kinase and its inhibitor SB; MARCKS, myristoylated alanine-rich C-kinase substrate; BAD, Bcl 2-associated death promoter.

totic pathways following Aβ or Aβ/Fe^{2+} addition to the neuronal cell cultures. In addition, we also evaluated the possible contribution of Fe^{2+} alone in unleashing pro-apoptotic signals in these cells.

As summarized in Fig. 9, our data indicate that micromolar concentrations of fresh preparations of Aβ peptide can stimulate PKC and Akt activities both of which are down-regulated by GF and wortmanin, respectively.

Of the principal downstream targets each one of these enzymes acts upon, we have chosen to study MARCKS and BAD protein phosphorylation. We recently characterized an $A\beta_{1-40}$-dependent phosphorylation of two MARCKS proteins of apparent molecular mass of 43 and 80 kDa in cerebral cultures (Kuperstein *et al*. 2001). When an anoxic stress was applied to cells, a reduction of the $A\beta_{1-40}$-dependent phosphorylation of MARCKS, most likely due to PKC down-regulation, was noticed.

In the present report, we examined Ser 136-BAD phosphorylation and provide solid evidence that like Akt, it highly depends on Aβ peptide addition. BAD is a pro-apoptitic member of the Bcl-2 family and one of the principal downstream substrates of Akt. Phosphorylation of BAD by Akt on Ser136, inhibits the pro-apoptotic sequel via the caspases cascade (Yuan and Yankner 2000) and Akt inhibitors such as wortmannin reduced BAD phosphorylation (Fig. 7). Enhanced BAD phosphorylability (Fig. 3) following Akt activation (Fig. 2) by soluble Aβ, is a feature possibly hinting to a role of the peptide in normal physiological functions and cell survival. This possibility is also in accord with a parallel reduction in p38 MAPK activity following Aβ treatment, a finding somewhat contradictory to other reports (Combs *et al*. 1999; Daniels *et al*. 2001). This discordance may be explained by the solubility state of Aβ since it has been shown that due to aggregation the peptide may undergo slow 'aging' (Barrow *et al*. 1992; Lorenzo and Yankner 1994) and formation of beta-pleated sheet secondary structures. In brief, the sequence of signaling events outlined above is in accord with the possibility that for as long as Aβ retains its solubility status, it does not lead to cytotoxicity.

Unlike treatment of cells with soluble Aβ alone, the combination of Fe^{2+} and $A\beta_{1-40}$ seems to initiate a different set of signaling cascades that concern both the ERKs and p38 MAPK family members. In a previous report, we showed that concomitant treatment with both compounds caused a high rise in the levels of ERKs that coincided with caspase-3 activation and cell death by apoptosis (Kuperstein and Yavin 2002). We also showed that the effect was blocked by the upstream MEK inhibitor U0126 and by the iron chelator DFe. In furthering these studies, p38 kinase, another family member of the MAPK superfamily and a putative signal indicative of cell death is strongly activated by the Aβ/Fe^{2+} combination (Fig. 4). Furthermore, treatment with SB an inhibitor of p38 MAPK, reverted the course of cell death as indicated by enhanced MTT reduction, lower numbers of TUNEL positive cells, enhanced BAD phosphorylation and prevention of caspase activation (Figs 5–8). Notable in this context is the down-regulation of PKC after Aβ/Fe^{2+} addition resembling in a sense the Aβ/anoxia-dependent PKC down-regulation previously documented (Kuperstein *et al*. 2001). The second aim of this study was to compare the signaling cascades generated by Aβ/Fe^{2+} and contrast them with those resulting from iron alone. In its various redox states, iron is on the one hand an essential component of normal cellular oxidation processes, but it may also act as a potential deleterious agent by stimulating hydroxyl radical formation (Boldt 1999). Previous (Kuperstein and Yavin 2002) as well as present results attest to the purported role of iron in inducing damaging processes such as formation of free radicals, activation of ERKs and p38 MAP kinases, overall enhancement of cellular dehydrogenase activity and activation of caspase-9 and -3 in neuronal cells. Also notable is, that independent of Aβ, divalent iron activates cellular signals involving anti-apoptotic signaling cascades such as PKC (Fig. 1) and Akt (Fig. 2). The biphasic time course characteristics of PKC isoforms modulation following iron addition, characterized by a rapid rise followed by a marked enzyme depletion, seems to follow a more complex pattern than that seen for Akt and p38 MAP kinases. The iron-induced increase in PKC levels, which to the best of our knowledge has not been previously shown, is in accord with enhanced PKC gene expression documented by others (Alcantara *et al*. 1994). The marked decrease in PKC levels subsequent to the early increase, is in accord with a blockade of iron-induced toxicity after addition of staurosporin and

K252 (Goodman and Mattson 1994), and is similar to that observed in neuronal cell cultures after anoxic stress (Kuperstein *et al*. 2001). The neurotoxic action induced by iron may require a disruption of the fine cellular oxidative state equilibrium (Boldt 1999), nevertheless this perturbation appears to be of transient nature. The short-lived nature of this process may well relate to a rapid change in the redox potential of divalent iron, as attested by the reduction of dichlorofluorescein stain (Kuperstein and Yavin 2002). The loss of iron redox activity may result from an irreversible change in its valence status or via binding and possible inactivation by other metal binding proteins. It may also explain both, reduction in lipid peroxidation and a higher proportion of cells surviving after treatment with iron (Kuperstein and Yavin 2002).

The ability to generate an Aβ/Fe^{2+} complex, as suggested by other laboratories (Huang *et al*. 1999; Atwood *et al*. 2000; Cuajungco *et al*. 2000), may enable the metal ion to retain its full redox capacity and carry out synergistic reactions on signaling cascades that could potentiate damage and enhance cell death. A typical example for this synergistic effect is the nearly complete loss of major PKC isoforms. Such losses as in the case of Aβ/Fe^{2+} addition (Fig. 1) or Aβ/anoxic stress share common grounds as they both aggravate damage and result in neuronal cell death. Furthermore from inferences from our previous study, which demonstrated a translocation of PKC to the membrane compartment following Aβ addition, we would like to postulate that either iron itself, or as a complex with Aβ, causes PKC translocation and subsequent down-regulation. Such losses in activity or losses in protein levels can be explained by a sensitivity of PKC to free radicals generated through Fenton chemistry (Traverso *et al*. 1996) via a process that is as yet unresolved.

The marked down-regulation of Akt after Aβ/Fe^{2+} addition paralleled BAD dephosphorylation. Furthermore, we have demonstrated that agents that inhibit PI3K/Akt cascade such as wortmannin, also caused a striking depletion of BAD phosphorylation (Fig. 7) and stimulated apoptosis (Fig. 6). Down-regulation of Akt and cell death that followed Aβ-induced toxicity have been attributed to an oxidative stress component by a number of laboratories (Zhang *et al*. 2001a,b; Han *et al*. 2001; Wang *et al*. 2000; Simakajornboon *et al*. 2001 Salinas *et al*. 2001). In accord with the work done by others, we also found that addition of antioxidants such as NAC and catalase markedly enhanced Akt activation and increased Ser 136-BAD phosphorylation (data not shown), thereby suppressing oxidation. In conclusion, the oxidative stress component in the experimental model outlined above appears as a major factor in amyloid beta induced cell damage. The balance between the levels of certain transition metals, Aβ peptide availability, free radicals production and oxidized lipids may however, determine the severity of the insult. The present studies provide us with a clue as to the types of signaling cascades that are initiated and those that are suppressed following the early stages of onset.

Gaining such information may be of potential relevance in the understanding of AD pathophysiology (Behl 1997, 2000; Lynch *et al*. 2000; Smith *et al*. 2000; Varadarajan *et al*. 2000). Indeed clinical studies suggest that increased oxidative stress is an early event that causes Aβ lesions formation (Nunomura *et al*. 2001), while brain trauma is believed to accelerate AD progression (Uryu *et al*. 2002). Oxidative stress caused by traumatic events such as stroke and ischemia has been associated with changes in levels of redox metals. Brain is particularly sensitive to oxidative stress because of the relatively high consumption of oxygen, low levels of antioxidants, free radical-generating systems and multiple double bonds of polyunsaturated fatty acids as targets for lipid peroxidation. Whether iron or other transition metal ions such a copper are the culprit that causes Aβ toxicity still needs to be clarified. Furthermore, the ability to bind a metal ion would argue for an antioxidant role of Aβ as an iron chelator that predates peptide aggregation and its presumed pro-oxidant activity (Kontush 2001; Smith *et al*. 2002). A recent study showed that a sulfur-based free radical of methionine residue 35 could transfer the oxidation potential to Gly 33 onto the Aβ molecule rendering it toxic (Kanski *et al*. 2002). With respect to the iron-induced oxidative stress component, our data show that interruption of certain steps in kinase cascades or chelation of iron by DFe may halt the pro-apoptotic kinase signaling cascade and rescue neuronal cells. Therefore the search for appropriate agents that may sequester metal ions or use of antioxidants along with drugs inhibiting pathological signaling cascades, should offer a multiple approach for preventing and treating AD and oxidative stress related disorders.

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